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# Black Bullhead: An Indicator of the Presence of Chemical Carcinogens

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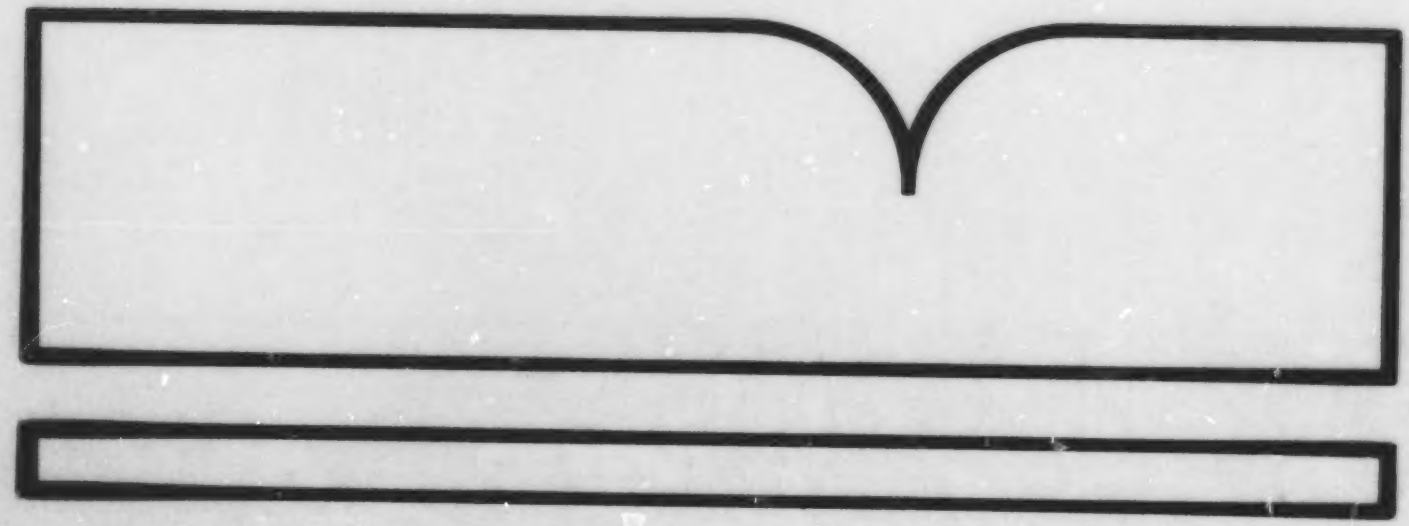

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16. ABSTRACT

The usefulness of exposing caged fish to polluted water to detect chemical carcinogens was demonstrated by the development of neoplasms on black bullheads. Chemical analysis in this case failed to indicate that mutagenic or carcinogenic chemicals were present, perhaps because of infrequent sampling. Laboratory exposures of black bullheads to sediment extracts (unpublished data) and brown bullhead embryos to organic extracts of the Tuskegee wastewater failed to cause tumors or other lesions that occurred on fish exposed in cages. Additional refinement of techniques may improve the usefulness of laboratory exposures; however, field exposure of caged fish is currently most satisfactory for the conditions and the carcinogen(s) present in the Tuskegee wastewater.

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# CHAPTER 36

# Black Bullhead: An Indicator of the Presence of Chemical Carcinogens

John M. Grizzle

Fish are useful indicators of pollutants in the aquatic environment because they accumulate and respond to toxicants. The ability of fish to develop neoplasms after exposure to carcinogens, their relatively short tumor-induction time, and the lower cost of carcinogenicity studies with fish than with mammals indicate that fish have potential for routine use as laboratory animals in carcinogenesis studies.[1,2] Several types of fish tumors can be induced by chemical carcinogens,[3] and the occurrence of tumors in wild fish has been used as evidence for the presence of carcinogenic substances in aquatic environments.[4,5] However, in some cases, viral or genetic causes cannot be eliminated because of uncontrolled conditions.

The presence of a chemical carcinogen in the chlorinated effluent entering the final oxidation pond of the south Tuskegee, Alabama, wastewater treatment plant was first suspected because black bullheads, *Ictalurus melas*, in this pond had a 73% prevalence of oral papillomas and indications of mixed function oxidase (MFO) induction.[6,7] Although papillomas are a common type of fish neoplasm,[8] those in this case were distinctive because of the high prevalence and because most were located in the oral fornices rather than in variable locations. The enzyme induction and high prevalence of papillomas in black bullheads from the final oxidation pond receiving chlorinated wastewater (no tumors or enzyme induction were found in fish from unpolluted ponds nearby) suggested that the tumors were related to the effluent entering the pond. However, analysis of water and sediment from the pond did not indicate the presence of toxicants considered capable of causing the papillomas.[6,9] These findings suggested three possibilities. First, that the papillomas were caused by a chemical carcinogen not detected by water analysis; second, that the papillomas were related to a tumor virus; or third, that the papillomas were genetically transmitted.

Because fish in this pond were confined and water was from a single source, the final oxidation pond offered an opportunity for experiments involving fish as indicators of the presence of chemical carcinogens. Black bullheads are



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suited for chronic exposure to chlorinated effluents because of their relatively high tolerance to chlorine. The 96-h $LC_{50}$ for black bullheads is 1.41 mg/L compared with 0.156 mg/L for channel catfish, *Ictalurus punctatus*, and 0.172 mg/L for rainbow trout, *Salmo gairdneri*.[10]

Several approaches, in addition to chemical analysis of the water, were used to study the cause of the papillomas on black bullheads in the wastewater pond. Studies were conducted with both caged fish in controlled experiments and fish free in the pond. This chapter summarizes the oncology at this wastewater treatment facility and illustrates the usefulness of field studies with black bullheads and other fish species for detecting carcinogens.

## STUDY SITE

The south Tuskegee wastewater treatment facility, located 7 km southwest of Tuskegee, Alabama, began operating during January 1977. This plant receives only domestic wastewater; there is no known discharge from industries or other sources suspected of releasing carcinogens. However, surface runoff from surrounding agricultural and residential areas probably enters the sewage system after heavy rainfall.

The 0.8-ha final oxidation pond was designed for postsecondary wastewater treatment. Before entering the final oxidation pond, wastewater undergoes activated sludge treatment, sedimentation, and disinfection by chlorination. Four mechanical aerators in the pond prevent stratification and maintain dissolved oxygen near 100% saturation. The pond has an earthen bottom with a concrete apron at the water-surface line and is surrounded by a dike that prevents runoff drainage from entering. At the average flow rate of $4 \times 10^6$ L/d, water retention time is approximately 5 d.

During January to November 1979, monthly averages of residual chlorine concentrations ranged from 1.3 to 3.1 mg/L in the effluent leaving the chlorine contact chamber and entering the final oxidation pond. Since November 1979, the chlorine concentration has ranged from 0.25 to 1.2 mg/L. The residual chlorine concentration of water leaving the pond was usually 0.1 mg/L, approximately the same as before November 1979.

Organic extracts of the pond water, tested with the Ames test, were mutagenic to bacterial tester strains TA98 and TA100, but only after metabolic activation by Aroclor-induced rat liver microsomes.[9] The acidic fraction of the wastewater was more mutagenic than the basic fraction, and the highest mutagenic activity occurred during the summer.

The only species of fish present in the final oxidation pond before May 1980 were black bullhead, green sunfish (*Lepomis cyanellus*), and golden shiner (*Notemigonus crysoleucas*). During May and July 1980, a total of 2000 silver carp (*Hypophthalmichthys molitrix*) was released in the pond.

## METHODS

The prevalence of tumors in wild fish from the final oxidation pond was determined in samples of 20 to 106 specimens from ten collections between November 1979 and March 1983. Gill nets were used for capturing most fish, but on one occasion, moribund fish were collected with a dip net. Most fish were examined grossly for external lesions. Selected fish were necropsied, and representative lesions were examined by light and electron microscopy. Fish were returned to the pond if they were not necropsied or used in experiments.

Black bullheads, brown bullheads (*Ictalurus nebulosus*), yellow bullheads (*Ictalurus natalis*), and channel catfish were placed in cages in the final oxidation pond and in a control pond at the Alabama Agricultural Experiment Station. The 1-$m^3$ cages were constructed of plastic netting supported at the top and bottom by steel hoops. Mesh size was 6 or 19 mm, depending on the size of the fish. Cages were allowed to rest on the pond bottom (sinking cages) or suspended off the bottom by floats (floating cages). Cages were placed at three locations in the final oxidation pond: near the inlet (inlet A), approximately 60 m from the inlet (inlet B), and near the pond outlet (outlet). Most cages were stocked with 50 fish of a single species, with total lengths of individual fish between 5 and 10 cm. All caged fish were fed a commercial catfish ration. Average time between examinations of caged fish for oral lesions was 74 d.

## RESULTS AND DISCUSSION

### Wild Fish

The prevalence of papillomas in wild, adult black bullheads in the final oxidation pond decreased from 73% in 1980 to 23% in March 1983. The size of papillomas also decreased with time, and the large papillomas seen protruding from the mouth of black bullheads in 1979–80[6] were no longer present. A relationship between chlorination and the black bullhead papillomas was indicated by the reduced prevalence of tumors after the chlorination rate was reduced. Tumors were not found in any species other than black bullhead.

Examination of black bullhead papillomas with transmission electron microscopy failed to reveal virus-like structures. Attempts to demonstrate a viral cause by injecting black bullheads with cell-free homogenized papillomas or by cell-culture techniques did not provide evidence that viruses were associated with the papillomas.[6,9] The inclusion bodies and electron-dense 35-nm particles in epithelial cells of some tumors examined ultrastructurally[6] probably do not indicate a viral cause of these papillomas, because cells with these inclusions and particles were not consistently present, and the 35-nm particles do not resemble any known tumor virus. If viruses are associated with the black bullhead papillomas, they are unlike those previously reported in fish neoplasms.[11]

## Caged Fish

The most common type of lesion that developed on fish confined to cages in the final oxidation pond was mucosal hyperplasia (sometimes accompanied by stomatitis) in the oral fornices (Table I). The location of these lesions was the same as the usual location of papillomas on wild black bullheads. Characteristics of these hyperplastic lesions that distinguished them from papillomas were their smooth surface, mucosal thickness less than 1 mm, and lack of intermixing of the mucosa and submucosa. Histologically, these lesions were similar to the epidermal hyperplasia that developed on brown bullheads painted with an extract of Buffalo River sediment; papillomas later developed on these brown bullheads.[12,13]

In addition to histological appearance, the nonneoplastic nature of hyperplastic lesions on caged fish was indicated by their relatively quick disappearance from the fish (Figure 1). In black bullheads stocked during October 1980 and February 1981, hyperplastic lesions developed during the first 2 to 6 months after the fish were placed into the pond, then most or all of the lesions regressed during the next 1 to 2 months. The development and regression of hyperplastic lesions in the first two groups of black bullheads stocked occurred two to three times in each group. Other species and black bullheads stocked later developed lower prevalences of hyperplastic lesions, which usually reached a maximum prevalence after most of the fish stocked in a cage had died (Table I). Therefore, the pattern of lesion development and regression could not be determined because of the low number of fish with lesions.

**Table I. Maximum Prevalence of Oral Mucosa Hyperplasia and Papillomas on Caged Fish in the Final Oxidation Pond of the Tuskegee, Alabama, Wastewater Treatment Facility**

| Species | Stocking Date | Hyperplasia | | Papillomas | | Control[a] |
|---|---|---|---|---|---|---|
| | | % | N[b] | % | N | % |
| Black bullhead | 21 Oct 80 | 95 | 22 | 80 | 5 | 4 |
| | 23 Feb 81 | 50 | 12 | 0 | | |
| | 29 Oct 81 | 33 | 6 | 0 | | 0 |
| | 9 Apr 82 | 25 | 8 | 0 | | |
| | 8 Jul 82 | 17 | 12 | 8 | 12 | 9 |
| Yellow bullhead | 12 May 81 | 0 | | 0 | | 14 |
| Brown bullhead | 29 Oct 81 | 0 | | 0 | | 0 |
| Channel catfish | 16 Jan 81 | 30 | 10 | 0 | | 8 |
| | 29 Oct 81 | 4 | 28 | 0 | | 4 |
| | 8 Jul 82 | 0 | | 0 | | 5 |

[a]Control is the maximum prevalence of hyperplastic oral lesions in caged fish kept in a control pond. Neoplasms did not occur in control fish.
[b]Number of samples on the date when the maximum prevalence occurred.

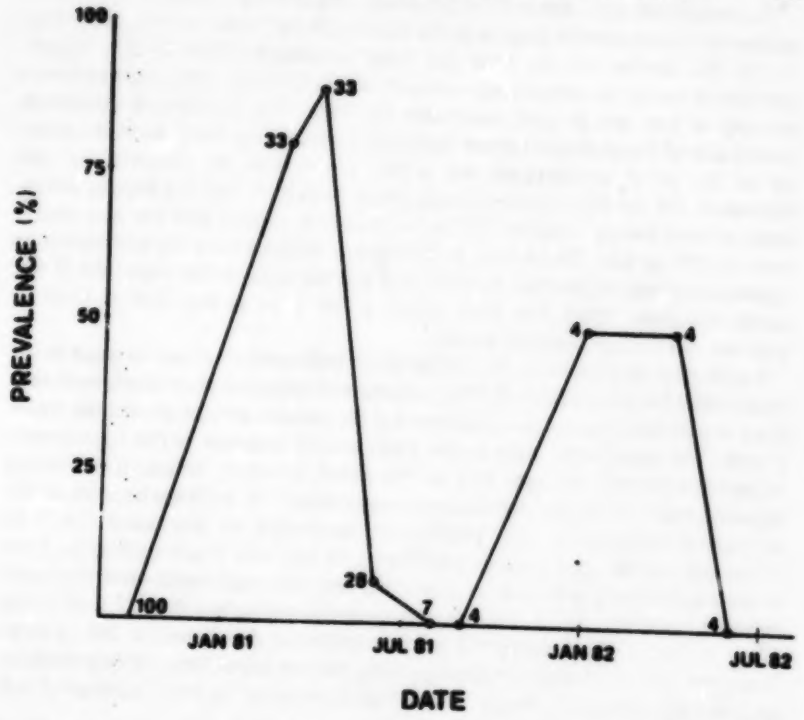


Figure 1. Prevalence of oral mucosa hyperplasia in black bullheads confined to floating cages stocked on October 21, 1980, near the outlet of the final oxidation pond of the Tuskegee, Alabama, wastewater treatment plant. The occurrence of lesions in this group of fish is representative of the pattern of lesion development and regression on most groups of black bullheads stocked on October 21, 1980, and February 23, 1981. The number at each data point indicates number of samples.

Attempts to correlate the prevalence of hyperplastic lesions with the season of the year or periods of high mutagenic activity in organic extracts of the wastewater were not successful. The lack of correlation between these lesions and mutagenicity may indicate a variable induction period, perhaps because of varied toxicant concentration or environmental conditions, or because different chemicals were involved with the lesions and mutagenicity. More frequent observation of the fish would have been helpful in better defining the length of time required for lesion development and regression, and might have made the influence of other factors (e.g., temperature, rain, short-term variation in chlorine concentration, or variation in the quantity of wastewater being treated) more evident.

The maximum prevalence of hyperplastic oral lesions on caged fish during exposures of comparable lengths in the final oxidation pond varied, depending on the fish species and the time that exposure began (Table I). The highest prevalence was in black bullheads stocked during October 1980, and maximum prevalence was less in each successive stocking. The decrease in maximum prevalence of hyperplastic lesions indicates a continuing decrease in the toxicity of the pond environment for a few years after the chlorination rate decreased. Of the four species tested, black bullheads had the highest prevalence of oral lesions. Similar lesions occurred on control fish but at a prevalence of 9% or less. There was no significant difference in the prevalence of hyperplastic lesions between floating and sinking cages or between inlet B and outlet locations. Most fish died within a few days at the inlet A location because of the high chlorine levels.

Papillomas developed on five of the black bullheads confined to cages in the final oxidation pond (Table I). The induction of papillomas on black bullheads from a different population indicates that the tumors are not genetically transmitted. The papillomas were in the same mouth location as the hyperplastic lesions developing on caged fish in this pond; however, lesions intermediate between hyperplasia and neoplasia were not observed, perhaps because of the infrequent observation. The papillomas developing on the caged fish were indistinguishable from smaller papillomas on the wild black bullheads. Four of the papillomas developed on caged fish near the pond outlet after they were exposed for 467 to 537 d. One papilloma developed in less than 57 d on a fish caged at the inlet B location. All of the papillomas developed on fish in cages that were in contact with the pond bottom, but the importance of cage location and contact with the sediment is uncertain because of the small number of fish that developed papillomas.

The reason for the uniform location of papillomas in black bullheads and hyperplastic lesions in caged ictalurids may be related to the presence of the oral valve in these species. The oral valve is a thin fold of mucosa located posterior to the upper and lower lips. The lateral ends of the valve are near the oral fornices, which were the most common locations of lesions. The oral valve is the only morphological distinction of the area where most lesions occur, but the reason for the sensitivity of the mucosa in this area has not been determined.

The usefulness of exposing caged fish to polluted water to detect chemical carcinogens was demonstrated by the development of neoplasms on black bullheads. Chemical analysis in this case failed to indicate that mutagenic or carcinogenic chemicals were present, perhaps because of infrequent sampling. Laboratory exposures of black bullheads to sediment extracts (unpublished data) and brown bullhead embryos to organic extracts of the Tuskegee wastewater[14] failed to cause tumors or other lesions that occurred on fish exposed in cages. Additional refinement of techniques may improve the usefulness of laboratory exposures; however, field exposure of caged fish is currently most satisfactory for the conditions and the carcinogen(s) present in the Tuskegee wastewater.

In other situations, laboratory exposures have been important in demonstrating the association between fish tumors and chemical carcinogens associated with pollution. Brown bullhead skin exposed to an organic extract from Buffalo River sediment developed epidermal papillomas, one of the types of neoplasms occurring in wild brown bullheads from the Niagara River and Lake Erie, which receive water from the Buffalo River.[12,13] The induction of papillomas during controlled exposures supports the hypothesis that tumors on wild fish from polluted environments can result from the pollutants, and that chemical carcinogens can cause fish papillomas.

The caged fish in the Tuskegee final oxidation pond were also useful for determining enzyme induction. Strength[15] determined that glucuronosyltransferase (UDP-GT) activity in caged channel catfish from the pond was higher than in controls. UDP-GT in other caged species, including black bullheads, and sulfotransferase activity in all caged species were not consistently different than controls. The induction of UDP-GT in channel catfish is similar to induction that occurs in rats and fish given oral or intraperitoneal doses of carcinogens[16-18] and is probably important in the excretion of carcinogens.

## CONCLUSIONS

Field exposure of fish to effluents appears to be a useful method for detecting carcinogens. During field exposures, fish can be examined for lesions, enzyme induction, or other effects of carcinogens. Direct field exposure is advantageous because extraction of the carcinogen from the effluent is not necessary, and exposure levels correspond to those actually present in the environment. The duplication of some field conditions, such as complex mixtures of chemicals at variable concentrations, could not be accomplished in laboratory experiments.

Chemical analysis and laboratory exposures of fish to carcinogens extracted from water have the disadvantage of, in most cases, requiring collection of water samples that may miss the intermittent presence of toxicants. Field exposures ensure that fish are exposed to carcinogens with intermittent presence. Exposed fish can accumulate some carcinogens, and those accumulated are evidence of exposure. Furthermore, carcinogens extracted from fish can be used to identify those that have been present in the water.

Tumor induction in fish is more conclusive evidence than in vitro tests for the presence of a carcinogen that has human-health implications, because carcinogenesis in vertebrate test animals is more directly related to human cancer. However, in vitro tests have the advantages of lower cost, quicker results, and, in some cases, of indicating something about the carcinogen, such as the type of mutation resulting from exposure. The Ames test was useful in demonstrating the presence of a mutagen in organic extracts of the Tuskegee wastewater, but the development of tumors on experimentally exposed fish was more convincing evidence for the presence of a carcinogen.

The use of cages for containment of test fish permits frequent observation as well as confinement to the location of interest. Observation or sampling of test fish is necessary to detect lesions, enzyme induction, or other changes, and to ensure that indirect results of effluent exposure, such as infectious diseases, are detected. The confinement of fish to a specific location prevents them from avoiding the effluent and permits a better determination of the extent of exposure. Confinement is especially important when the fish could easily leave the area of interest.

The disadvantages of field exposures include more difficult observation of lesion development and less control over exposure concentration and environmental conditions than in laboratory experiments. In many polluted environments, the chances that test fish have for surviving chronic exposures are low because of periods of lethal conditions. Field exposures may also subject the fish to toxicants other than those of interest; therefore, the results are more difficult to interpret. Although the response time for tumor induction is long compared to chemical analysis of water or in vitro tests, the use of preneoplastic lesions or metabolic changes may be useful in reducing the time required for obtaining results from fish exposures.

In polluted environments that are not suitable for chronic fish exposures, field exposures may be useful for determining acute effects but would not be useful for demonstrating carcinogenic effects. In these cases, the extraction of suspected carcinogens and subsequent laboratory exposure would be more appropriate than field studies. It is probable that the Tuskegee pond would not have been suitable for field exposures if the pond had not been aerated as part of the treatment process. In some environments that cannot support fish life, artificial aeration or other changes might allow fish to live, but possible changes in the toxicants of interest must be considered.

The most desirable species for field exposures depends on the situation being studied. The selection of a test species must consider the environmental conditions, the nature of the toxicant, and the objectives of the experiment. When there is no compelling reason for selecting a particular species, choosing one for which there is basic information, such as culture requirements and sensitivity to toxicants, will improve the chances of a successful experiment. The survival time of exposed fish must be long enough for the desired response to occur. In many polluted aquatic environments, factors such as dissolved oxygen, pH, or toxicant concentrations are too adverse for chronic exposures of some or all fish species.

In the case of the Tuskegee wastewater pond, the black bullhead was an obvious choice for experimental exposures because of the occurrence of papillomas on wild black bullheads. This species would probably be desirable for other experimental situations because of its tolerance to pollution, wide geographical occurrence,[19] and ease of culture. Its relatively high tolerance to chlorine is an advantage for chronic exposure to chlorinated effluents that exceed the tolerance of other species. Although the reasons for the differences are not yet known, black bullheads seem more susceptible to the carcinogenic

effects of the chemical carcinogen(s) in the Tuskegee wastewater pond than other exposed species.

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